Paternal Transmission of Stress-Induced Pathologies

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Background: There has been recent interest in the possibility that epigenetic mechanisms might contribute to the transgenerational transmission of stress-induced vulnerability. Here, we focused on possible paternal transmission with the social defeat stress paradigm.

Methods: Adult male mice exposed to chronic social defeat stress or control nondefeated mice were bred with normal female mice, and their offspring were assessed behaviorally for depressive- and anxiety-like measures. Plasma levels of corticosterone and vascular endothelial growth factor were also assayed. To directly assess the role of epigenetic mechanisms, we used in vitro fertilization (IVF); behavioral assessments were conducted on offspring of mice from IVF-control and IVF-defeated fathers.

Results: We show that both male and female offspring from defeated fathers exhibit increased measures of several depression- and anxiety-like behaviors. The male offspring of defeated fathers also display increased baseline plasma levels of corticosterone and decreased levels of vascular endothelial growth factor. However, most of these behavioral changes were not observed when offspring were generated through IVF.

Conclusions: These results suggest that, although behavioral adaptations that occur after chronic social defeat stress can be transmitted from the father to his male and female F1 progeny, only very subtle changes might be transmitted epigenetically under the conditions tested.

M ajor depressive disorder (MDD) is among the foremost causes of disability in the world (1). It is an extremely common disorder, with an overall lifetime risk estimated to be approximately 15% in the general US population, and is thought to be caused by a combination of genetic and environmental factors (2). A rich literature has demonstrated that MDD is highly heritable, with roughly 40% of the risk being genetic (3,4). However, specific causative genes have been difficult to identify with certainty. Environmental factors also contribute importantly. For example, life-time exposure to any of several forms of chronic stress dramatically increases the risk of an individual for MDD (5,6).

In recent years, there has been increased attention to the possibility that a third mechanism might also contribute to MDD, namely, epigenetics. By definition, epigenetics refers to any changes in organismal or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence (genotype). Accordingly, exposure to environmental stress might produce epigenetic alterations (for example, via DNA methylation) in germ cells, which might then affect the phenotype of offspring to influence inherent vulnerability to MDD. Recent insight into this provocative possibility has come from rodent models. Repeated early life stress (maternal separation) has been shown to cause lifelong alterations in depression- and anxiety-like behavior and in alterations in the hypothalamic-pituitary-adrenal axis (7). There also is a growing literature that such early life stress increases the vulnerability of offspring of these animals to stress (8,9). This transgenerational transmission of stress vulnerability seems to be behaviorally transmitted (i.e., it is mediated by types of maternal behavior that can pass from one generation to the next). Interestingly, however, a recent study demonstrated that such stress vulnerability might also be transmitted epigenetically by changes in germ cells that subsequently influence the behavior of the offspring for up to three generations in a gender-dependent manner (10).

Here, we used the chronic social defeat stress paradigm in adult male mice to further investigate the transgenerational transmission of stress vulnerability, in this case focusing on paternal transmission. We have demonstrated previously that chronic social defeat stress induces several measures of depression- and anxiety-like abnormalities, some of which are very stable and can be reversed by chronic but not acute treatment with standard antidepressants (11–14). We studied whether exposure to chronic social defeat stress causes stress-related abnormalities in the F1 offspring of the stressed fathers and whether such transmission might be mediated epigenetically by use of in vitro fertilization (IVF). To further understand a biological mechanism for the transmission of depressive-like behaviors, we examined putative biomarkers of depression in humans—namely, plasma levels of corticosterone and vascular endothelial growth factor (VEGF)—in offspring of nondefeated and defeated mice (15,16). Our results demonstrate that, although relatively strong behavioral abnormalities can be transmitted to the next generation, most are not transmitted by IVF, suggesting a limited role for epigenetic mechanisms.

Methods and Materials

Animals

All experimental C57Bl/6J male and female mice (7 weeks) were obtained from the Jackson Laboratory (Bar Harbor, Maine). Retired CD1 breeders used as the aggressors for the social defeat paradigm were obtained from Charles River (Wilmington, Massachusetts). All mice were maintained in a temperature- and humidity-controlled facility on a 12-hour light–dark cycle with food and water ad libitum. All procedures were carried out in accordance with the Insti-

tutional Animal Care and Use Committee guidelines of the Mount Sinai School of Medicine.

Social Defeat

Chronic social defeat stress was performed exactly as previously published (11,13,14,17). Briefly, male C57 mice were exposed to a novel aggressive CD1 male mouse for 10 min/day, after which the mice were separated by a Plexiglas barrier that allows for sensory contact without further physical interaction. Control mice were housed two animals/cage under the same conditions as their experimental counterparts but without the presence of an aggressive CD1 mouse. Twenty-four hours after the last of 10 daily defeat or control episodes, the fathers used for all experiments (including IVF) were evaluated in a social interaction test to assess social avoidance.

Breeding

In the first experiment, male C57 mice were subjected to 10 days of social defeat stress and, 1 month later, were paired along with control nondefeated mice with a normal C57 female for 10 days, at which time the male was removed. In a separate experiment (pre/postoffspring study), normal males were bred with a female; the offspring of such pairings were labeled "predefeat." The males were then exposed to 10 days of defeat stress and, 1 month later, were paired with a female; the offspring of these pairings were labeled "postdefeat." Because we selected defeated fathers that have a susceptible phenotype, for comparison we also included one generation of offspring of control mice that were not subsequently selected for any depressive traits, therefore representing a random representation of C57 mice. This allowed us to test directly whether the transmission of behavioral abnormalities is a result of any pre-existing (i.e., latent) factors that might exist in this inbred mouse line. Because the depressive-like symptoms after social defeat are known to be extremely stable for at least 1 month (11,13), all breeding was initiated 1 month after social defeat stress to focus on longer-lasting adaptations. Future experiments are required to learn whether different time points after defeat would result in different findings.

Behavioral Testing

Offspring of defeated and nondefeated mice were also evaluated in a battery of behavioral tests in the following order: elevated-plus-maze, response to novel environment, sucrose preference, and forced swim test. A separate group of mice were not subjected to the forced swim test and instead were studied for their susceptibility to a submaximal course of social defeat stress as noted in the following text.

All behavioral tests were carried out as described previously (13,18). In the elevated plus maze, mice were placed in the center of the maze, and behavior was recorded by videotracking for 5 min under total darkness. Time spent in each zone was analyzed with Ethovision software (Noldus, Utrecht, the Netherlands). Responses to a novel environment were measured by placing the mouse in an unfamiliar chamber for 15 min. Locomotor activity was recorded automatically by infrared photobeams. For sucrose preference, animals were acclimated for 3 consecutive days to two-bottle choice conditions (water or 1% sucrose) before additional days of choice testing with the position of the tubes interchanged daily. Sucrose preference was calculated as a percentage of sucrose over total liquid consumed and was averaged over the 3 test days. A 1-day forced swim test was conducted for a period of 5 min, and latency to immobility was measured.

To assess susceptibility to social defeat stress, we used a submaximal defeat paradigm that does not produce social avoidance in control mice but reveals prosusceptibility factors (13,19). Mice were exposed to a CD1 aggressor three times with 15-min intervals between each exposure. Twenty-four hours later, mice were assessed with the social interaction test.

Neuroendocrine Measures

Trunk blood was collected from mice at baseline or 5 min after 10 min of acute restraint stress, in ethylenediaminetetraacetic acid-lined tubes, and centrifuged at 3000 rpm for 20 min. Plasma was removed and stored frozen ($-80°C$) until analysis. Levels of corticosterone and of VEGF were measured with commercially available immunoassay kits (Immunodiagnositic Systems, Tyne and Wear, United Kingdom; and R & D Systems, Minneapolis, Minnesota, respectively) according to the directions of the manufacturers. For corticosterone, the intra-assay variability ranged from 3.8% to 6.6%, interassay variability ranged from 7.5% to 8.6%, and mean assay sensitivity was .55 ng/mL. For VEGF, intra-assay variability ranged from 4.3% to 8.4%, interassay variability ranged from 5.7% to 8.4%, and mean assay sensitivity was 3.0 pg/mL. All blood and was collected during the lights-on phase between 2:00 PM and 5:00 PM.

IVF

We performed IVF as routinely done by the facility at Mount Sinai Core Laboratory with an established protocol for rodent IVF (20). Fresh sperm from either control (nondefeated) or defeated (susceptible) mice was isolated from the cauda epididymis of behaviorally tested defeated and nondefeated control males. Bilateral cauda epididymides with several millimeters of attached vas deferens were isolated into 1 mL of Cook's Research VitroFert media (K-RVFE-50; Cook, Brisbane, Australia) in a 35-mm tissue culture dish. With two 26-gauge tuberculin syringe needles, several cuts were made across the epididymis, and any sperm in the attached vas deferens was pushed out the cut end with the syringes. The dish was placed in a 37°C oven for 10 min to allow the sperm to be released from the cut epididymis into VitroFert media. With wide-bore pipette tips to prevent damage to the sperm, 20–40 mL of the sperm suspension was added to 250 mL drops of VitroFert under mineral oil. One hour later, cumulus masses were isolated from superovulated donor females and placed into the VitroFert drops with the isolated sperm (cumulus masses from 3–5 females were placed into each drop). After 4–6 hours, the oocytes were recovered, washed through four changes of FHM media (MR-024-D; Millipore/Specialty Media, Phillipsburg, New Jersey), and cultured in microdrops of KSOM + amino acid media (Millipore/Specialty Media) overnight in a 5% carbon dioxide incubator. All 2-cell stage embryos were collected and transferred to the oviduct of pseudopregnant females, where they develop naturally to term.

Statistical Analysis

Two-tailed Student *t* tests were used to assess social avoidance of the fathers in the social interaction test after chronic social defeat stress. For the susceptibility of offspring to submaximal defeat, two-way analysis of variance (ANOVA) was performed, with breeding and submaximal defeat experience as the main factors. In the experiment where mice were bred both before and after defeat stress, statistical calculations were performed with one-way ANOVAs to confirm that there were no statistical differences between control and predefeat animals. This was then followed by a comparison of the predefeat and postdefeat mice with repeated measures ANOVAs with breeding
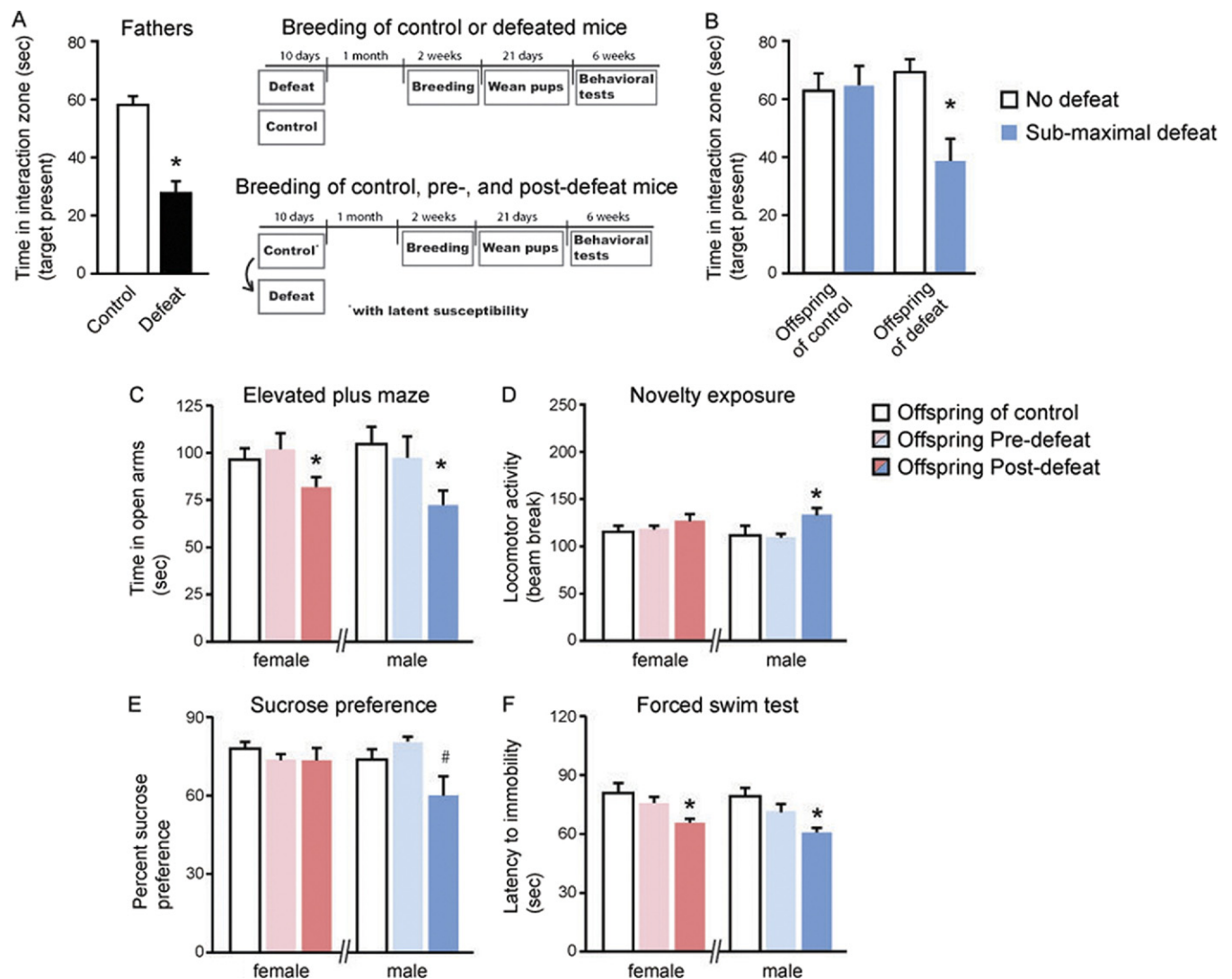
Figure 1. Paternal transmission of stress-induced depression- and anxiety-like behaviors via natural breeding. (**A**) Chronic social defeat induced robust social avoidance in male C57 mice, compared with control nondefeated mice (*$p < .05$). Experimental time line of how these mice were then bred with normal females for behavioral analysis of the offspring. (**B**) Offspring of socially defeated mice displayed robust social avoidance when exposed to a submaximal defeat paradigm (*$p < .05$), which produced no such social avoidance in the offspring of control mice. (**C–F**) Comparisons of offspring of control mice and offspring from fathers both before undergoing social defeat (predefeat) and after undergoing social defeat (postdefeat). (**C**) Male and female offspring of postdefeat offspring only showed an anxiogenic-like phenotype as measured by an increase in time spent in the open arms of the elevated plus maze (*$p < .05$). (**D**) Only the postdefeat male offspring showed an increase in locomotor behavior in a novel environment (*$p < .05$). (**E**) Likewise, in the sucrose preference test, only the postdefeat male offspring exhibited a trend toward a reduction in sucrose preference (#$p = .07$). (**F**) Both male and female postdefeat offspring showed a decrease in latency to become immobile in the forced swim test (*$p < .05$), compared with control and predefeat offspring. All values represent means ± SEM. All asterisks (*) are comparisons of predefeat and postdefeat offspring.

used as the main factor. Each gender was analyzed independently and followed up with appropriate post hoc tests. For the IVF experiment, planned comparison (*t*) tests were used to compare offspring from control and defeated mice derived through IVF. Statistical significance was set to a *p* value $< .05$.

Results

We first tested whether depressive-like behaviors induced in normal male C57 mice could be transmitted to their offspring. We used social avoidance as our primary measure, because this has proven to be a highly reliable and long-lived consequence of chronic social defeat stress, which is reversed by chronic (not acute) antidepressant treatment (11–13,21). As expected, 10 days of

chronic social defeat stress induced robust social avoidance when compared with nondefeated control mice ($n = 8$–9/group) [$t(15) = 2.229, p < .05$] (Figure 1A). One month later, defeated and nondefeated mice were bred with normal C57 female mice. The males were removed shortly after the females became pregnant. The male offspring were then studied, at 8–10 weeks of age, by subjecting them to a submaximal defeat paradigm that does not cause social avoidance in control mice but has been validated to reveal prosusceptibility factors (13,14). Male mice bred from defeated fathers but not from control fathers showed pronounced social avoidance in this paradigm (Figure 1B). Two-way ANOVA analysis revealed a main effect [$F(1,36) = 8.155, p < .05$], and further Tukey's Multiple Comparison testing revealed that the source of this effect was the decrease in interaction time of offspring of defeated mice

Table 1. Plasma Levels of Corticosterone and VEGF

	Male Offspring		Female Offspring	
	Predefeat	Postdefeat	Predefeat	Postdefeat
Corticosterone				
Basal	10.1 ± 1.64	25.3 ± 3.78^a	38.3 ± 7.51	48.5 ± 8.16
Stressed	$143 \pm .99$	162 ± 17.3	188 ± 12.6	195 ± 5.95
VEGF				
Basal	4249 ± 6.977	4185 ± 22.70^b	1755 ± 394.3	1498 ± 348.3
Stressed	4239 ± 7.030	4201 ± 20.24	1688 ± 512.2	988.1 ± 240.2

Absolute values with the SEM of plasma levels of corticosterone (mg/mL) and vascular endothelial growth factor (VEGF) (pg/mL) in predefeat and postdefeat male and female offspring, both at baseline and after an acute stress. Postdefeat male offspring exhibited an increase in basal corticosterone and a decrease in basal VEGF, with no differences seen after acute stress. In female offspring, there were no differences in corticosterone or VEGF levels under basal or stressed conditions.

[a]$p < .01$.
[b]$p < .05$.

exposed to submaximal defeat ($p < .05$). Previous work from our laboratory has demonstrated that a proportion (approximately 30%) of chronically defeated mice are resilient to most of the negative behavioral adaptations after defeat, including social avoidance (13). Surprisingly, there was no difference in the susceptibility of the offspring derived from resilient ($n = 7$) or susceptible ($n = 6$) mice, with both showing increased social avoidance to submaximal defeat (12.36 ± 5.67 sec and 15.74 ± 5.169 sec of interaction time, respectively).

To further investigate the role of paternal influence after chronic social defeat stress in depressive- as well as anxiety-like behaviors, we bred male C57 mice with normal female mice both before and after they were exposed to chronic defeat and later examined a battery of behaviors in the male and female offspring. Nondefeated control mice were also bred. We bred mice before social defeat to test the possibility that the increased vulnerability seen in the offspring did not arise from the defeat per se but rather from some pre-existing inherent factors. Unless otherwise noted, these experiments included control-bred male mice ($n = 9$), males from predefeat fathers ($n = 12$), males from postdefeat fathers ($n = 12$), control-bred female mice ($n = 8$), females from predefeat fathers ($n = 17$), and females from postdefeat fathers ($n = 17$). To compare the predefeat and postdefeat animals as nonindependent samples, we first confirmed for all behavioral tests in both male and female mice that there were no statistical differences between control and predefeat offspring ($p > .05$ for all comparisons). We then further analyzed offspring from the predefeat and postdefeat mice with repeated measures ANOVAs.

We assessed anxiety-like behaviors with two measures, the elevated plus maze and response to novelty. As depicted in Figure 1C, in the elevated plus maze for male offspring, there was a significant main effect of breeding [$F(1,11) = 14.791$, $p < .05$]. Male offspring from postdefeat fathers spent significantly less time in the open arms in comparison with predefeat offspring ($p < .05$). In the case of females there was a significant difference between predefeat and postdefeat female offspring [$F(1,16) = 6.0259$, $p < .05$], because female offspring from defeated fathers spent significantly less time in the open arm of the maze, compared with predefeat offspring ($p < .05$). We next examined the various groups of offspring for their response to novelty (Figure 1D), where increased locomotor activity is taken as a measure of anxiety-like behavior (20). In male offspring there was an overall effect of novelty [$F(1,11) = 10.275$, $p < .05$], which was due to an increase in locomotor activity in the offspring of postdefeat fathers ($p < .05$). There was no effect in response to novelty in female offspring ($p > .05$).

To test depression-like behaviors, we used the sucrose preference and forced swim tests, two widely used assays for screening depression-like symptoms in rodents (22). In male offspring, there was a strong trend toward a significant effect of breeding in the sucrose preference test [$F(1,11) = 3.940$, $p = .07$] (Figure 1E). In contrast, female offspring showed no effect of breeding condition in this test, with both predefeat and postdefeat offspring having similar sucrose preferences ($p > .05$). In the forced swim test (Figure 1F), we observed a main effect of breeding on latency to immobility in the male offspring [$F(1,11) = 4.939$, $p < .05$]. There was a significant decrease ($p < .05$) in latency in the offspring from postdefeat fathers in comparison with predefeat fathers (Figure 1F) ($p < .05$). A similar pattern was seen in female offspring [$F(1,16) = 5.680$, $p < .05$], because again there was a significant decrease in latency to immobility in the offspring from postdefeat fathers when compared with predefeat offspring ($p < .05$). Analysis of a separate group of control, predefeat, and postdefeat male offspring showed that only postdefeat offspring exhibited the increased susceptibility to submaximal defeat, as demonstrated in the initial experiment (Figure 1B).

In still another group of mice generated from predefeat and postdefeat fathers, we examined hormonal markers under baseline conditions and in response to acute restraint stress (Table 1). We examined plasma levels of corticosterone, a functional measure of the hypothalamic-pituitary-adrenal axis, a well-characterized component of stress responses (23). In males, there was a significant increase in plasma levels of corticosterone under baseline conditions in the postdefeat offspring ($n = 7$), compared with the predefeat offspring ($n = 9$) ($t = 3.513$, $p < .05$). However, no difference was observed after exposure to an acute stress ($n = 6-8$). No such differences were observed in the female offspring of predefeat and postdefeat fathers. We also examined plasma levels of VEGF, which has been implicated more recently in depression-related rodent models (24,25). Under baseline conditions, male offspring from postdefeat fathers ($n = 7$) had significantly lower VEGF levels compared with offspring of predefeat fathers ($n = 9$, $p < .05$), with no differences seen after acute stress. No difference in VEGF levels was seen in female offspring, although such measurements were highly variable in female mice.

In the final set of experiments, we used IVF to investigate whether the behavioral phenotypes observed in the aforementioned experiments were directly transmissible through the sperm of socially defeated mice. Sperm from defeated and control mice were used to impregnate normal female mice (Figure 2A). The offspring of these mice were later examined in the same battery of
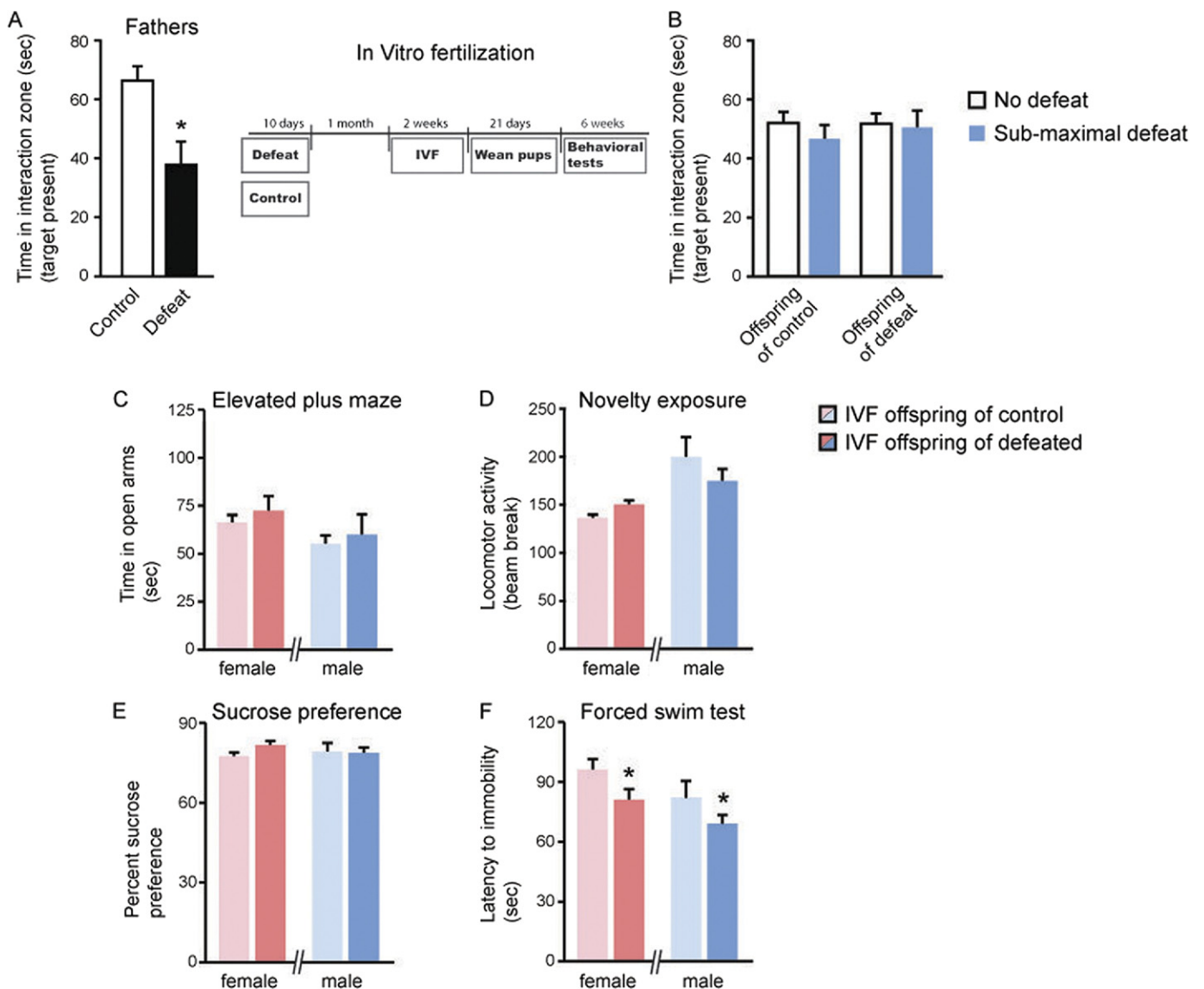
Figure 2. Paternal transmission of stress-induced depression- and anxiety-like behaviors via in vitro fertilization (IVF). (**A**) Chronic social defeat stress induced robust social avoidance in male C57 mice, compared with control nondefeated mice (*$p < .05$). Experimental time line including IVF and subsequent behavioral experiments. (**B**) Offspring derived from defeated or control fathers with IVF showed no social avoidance following a submaximal defeat paradigm. (**C**) The IVF defeated male and female offspring did not differ in time spent in the open arms of the elevated plus maze, compared with IVF control offspring. (**D**) There was a trend for increased locomotor activity in a novel environment in female IVF defeated offspring, compared with female IVF control offspring ($p = .06$) but no difference in males. (**E**) There was no difference in sucrose preference in either male or female IVF defeated offspring, compared with IVF control offspring. (**F**) In comparison with IVF control offspring, both male and female IVF defeated offspring showed a small but significant decrease in latency to become immobile in the forced swim test (*$p < .05$). All values represent means ± SEM.

behavioral tests as in our previous experiments. Unlike our previous findings, animals derived with IVF from defeated fathers were not more susceptible to a submaximal defeat paradigm compared with IVF control mice and showed no social avoidance (Figure 2B) ($n =$ 12–18/group, $p > .05$). We also found no significant difference between IVF control and IVF defeated offspring in time spent in the open arms of the elevated plus maze (Figure 2C) ($p > .05$). There was a trend toward increased locomotor activity in a novel environment in the IVF defeated compared with IVF control female offspring ($p =$ .06); however, no difference was apparent in the male offspring (Figure 2D) ($p > .05$). In the sucrose preference test, there was no effect when comparing IVF control with IVF defeated offspring of either gender (Figure 2E). Importantly, in the forced swim test, we

found that both male [$t(33) = 1.876, p < .05$] and female [$t(28) =$ 1.752, $p < .05$] IVF defeated offspring exhibited a small but significant decrease in latency to immobility, compared with IVF control mice (Figure 2F).

Discussion

Our data demonstrate that key aspects of both depressive- and anxiety-like behaviors can be transmitted to the offspring of socially defeated fathers. Although abnormalities were observed in both male and female offspring of stressed fathers, in general, a more robust phenotype was seen in the male offspring. That such behavioral abnormalities were seen only in offspring bred after the fathers

experienced defeat and not before indicates that the vulnerability being transmitted is a direct effect of the stress and not caused by some pre-existing diathesis. The male offspring of defeated fathers also exhibited a significant disturbance in neuroendocrine signaling, specifically, increased plasma levels of corticosterone and decreased levels of VEGF. These differences were observed at baseline, with no differences seen after an acute stress. Altered plasma levels of these two hormones are consistent with previous reports implicating corticosterone and VEGF in both depressive-like phenotypes and the efficacy of antidepressant treatment (23–25). Interestingly, although social defeat induces two distinct behavioral phenotypes, susceptible and resilient mice (13), offspring of these mice are equally vulnerable. This raises the intriguing question of the shared mechanism for this transmission. Of interest, we have previously demonstrated that, although by definition they do not display depressive-like features, resilient mice do display increased anxiety-like behavior comparable to that seen in susceptible individuals (13). This heightened anxiety might affect the care given by the mother to the progeny. However, further work is needed to explore this possibility. Additionally, because of the timing of breeding for the prepost experiment, we cannot definitively rule out that the behavioral abnormalities observed might be due to prior mating experience or age-dependent effects on gamete development. However, given our original observations in which similar differences were obtained in offspring of control and defeated mice of the same age and sexual experience, we believe that these caveats are not a major contributor to the behavior observations.

The transgenerational transmission of stress vulnerability traits in offspring of socially defeated mice implicates a role for epigenetic modifications in the paternal germ line. Indeed, a recent study reported the transgenerational transmission of stress vulnerability induced by maternal separation, a form of early-life stress, that is mediated through the male germ line (10). This study related such transgenerational transmission to altered DNA methylation at CpG islands of the MeCP2 (methylated CpG binding protein 2), CB1 (cannabinoid receptor 2), and CRFR2 (corticotropin release factor receptor 2) genes in the sperm of early-life stressed males (10). These provocative findings suggest that environmental perturbations can lead not only to lifelong behavioral adaptations for the individuals who experience such challenges (8,9) but might also alter behavioral responses of future generations who were not directly exposed to the traumas per se (10).

However, our data argue for more complex explanations, at least with respect to social defeat stress experienced during adulthood. That most of the transgenerational transmission of stress vulnerability observed in our experiments was not seen with IVF argues against the preponderance of epigenetic mechanisms. Rather, our data would suggest that the bulk of the vulnerabilities are passed on to subsequent generations behaviorally, presumably on the basis of the female detecting that she had procreated with an impaired male. Indeed, female rodents are known to adjust their reproductive investment, depending on the interaction that they had with the male (26). The exact mechanism behind such behavioral adaptations in the case of our results remains hypothetical. For example, precopulatory, copulatory, or postcopulatory behavior of the defeated male might cause increased female stress perhaps via direct physical aggression/interaction, pheromonal signaling, or ultrasonic vocalization, which could conceivably indicate inferiority or a degree of unfitness. It should also be noted that the process of IVF itself has several caveats that need to be considered. The IVF process might select sperm that are in different stages of maturation, which might have a direct impact on the level of genomic imprinting (27). Therefore, the lack of transmission of robust dis-

ease traits in our experiments might reflect epigenetic erasure. Moreover, IVF generated offspring did show statistically significant behavioral abnormalities, albeit far more subtle and limited than those observed with natural procreation, suggestive of a limited influence of true epigenetic mechanisms.

In summary, we have demonstrated the transmissibility of both depressive- and anxiety-like phenotypes to the F1 generation in male and, to a lesser extent, in female offspring of socially defeated male mice. Future studies are needed to examine whether these behavioral phenotypes are passed to subsequent generations (e.g., F2), as recent studies have demonstrated for early life stress, which can be transmitted several generations in a gender-dependent manner (10). Although our IVF experiments indicate that most of the transgenerationally transmitted behavioral phenotypes likely occur through behavioral mechanisms, a small role for epigenetic modifications is apparent, which now requires genome-wide assessments of chromatin changes in sperm of socially defeated mice and the elaboration of how any such changes observed lead to differences in neurobiology and behavior in the offspring.

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